MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: antibodies to ATP6V0D1, ATP6V1B2 and LAMP2 from Abcam; antibody to ATP6V0c from Novus Biologicals; antibody to ATP6V1D from Santa Cruz Biotechnology; antibody to ATP6V1A from GeneTex; antibody to raptor from Upstate/Millipore; HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1, RagA and RagC, mTOR, raptor, phospho-T398 dS6K, phospho-S473 Akt, Akt, and the FLAG epitope from Cell Signaling Technology; antibodies to the HA and Myc tags from Bethyl laboratories; antibody to Rheb was a generous gift from Paul Worley (Johns Hopkins University). RPMI, FLAG M2 affinity gel, ATP, cycloheximide, amino acids, amino acid esters and Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) from Sigma Aldrich; protein G-sepharose and immobilized glutathione beads from Pierce; DMEM from SAFC Biosciences; FuGENE 6 and Complete Protease Cocktail from Roche; Alexa 488 and 568-conjugated secondary antibodies; 70KDa dextran-Oregon Green 514; Schneider's media, Express Five Drosophila-SFM, and Inactivated Fetal Calf Serum (IFS) from Invitrogen; amino acid-free RPMI, and amino acid free Schneider's media from US Biological; Concanamycin-A from A.G. Scientific. The dS6K antibody was a generous gift from Mary Stewart (North Dakota State University). Salicylihalamide A was a generous gift from Jeff De Brabander (UT Southwestern).

Cell lysis and immunoprecipitation

HEK-293T cells stably expressing FLAG-tagged proteins were rinsed once with ice-cold PBS and lysed in ice-cold lysis buffer (150mM NaCl, 40 mM HEPES [pH 7.4], 2 mM EGTA, 2.5mM $MgCl_2$, 1% Triton X-100, and one tablet of EDTA-free protease inhibitors (Roche) per 25 ml). The soluble fractions from cell lysates were isolated by centrifugation at 13,000 rpm for 10 minutes in a microfuge. For immunoprecipitations, 35 μl of a 50% slurry of anti-FLAG affinity gel (Sigma) were added to each lysate and incubated with rotation for 2-3 hours at 4°C. Immunoprecipitates were washed three times with lysis buffer. Immunoprecipitated proteins were denatured by the addition of 35 μl of sample buffer and boiling for 5 minutes, resolved by 8%–16% SDS-PAGE, and analyzed by immunoblotting.

A similar protocol was employed when preparing samples for mass spectrometry.

Mass spectrometry

Immunoprecipitates from HEK-293T cells stably expressing FLAG-p18, FLAG-p14 and FLAG-RagB were prepared as described above. Proteins were eluted with the FLAG peptide from the anti-FLAG affinity beads or recovered by boiling with sample buffer, resolved on 4-12% NuPage gels (Invitrogen), and stained with simply blue stain (Invitrogen). Each gel lane was sliced into 10-12 pieces and the proteins in each gel slice digested overnight with trypsin. The resulting digests were analyzed by mass spectrometry as described (*1*). Between 3 and 57 peptides corresponding to various v-ATPase subunits were identified in the FLAG-p18, FLAG-p14 and FLAG-RagB immunoprecipitates, while no peptides were detected in negative control samples, which included lysates from HEK-293T cells stably expressing FLAG-Rap2a or a FLAG-tagged mRFP targeted to mitochondria via fusion to the transmembrane domain of OMP25 (*2*).

In vitro protein binding assays

HEK-293T cells plated the previous day at a density of 10^6 per 15cm Petri dish were transfected separately with FLAG-tagged Ragulator subunits or HA-GST-tagged v-ATPase subunits. 48 hrs post-transfection, cells were lysed in 2ml of ice-cold lysis buffer (150mM NaCl, 40 mM HEPES [pH 7.4], 2 mM EGTA, 2.5mM $MgCl_2$, 1% Triton X-100, and one tablet of EDTA-free protease inhibitors [Roche] per 25 ml). Soluble fractions were immunoprecipitated with 150 µl of a 50% slurry of anti-FLAG affinity beads (Sigma) to isolate FLAG-tagged Ragulator subunits, or 150 µl of a 50% slurry of immobilized glutathione affinity beads (Pierce) to isolate GST-tagged v-ATPase subunits, for 2h at 4°C. Samples were washed once in lysis buffer, followed by 3 washes in lysis buffer supplemented with 500mM NaCl. Lysis buffer was then replaced with binding buffer (40 mM HEPES [pH 7.4], 2 mM EGTA, 2.5mM $MgCl_2$, 0.3% CHAPS); FLAG-tagged proteins were eluted from the affinity beads using a competing FLAG peptide (sequence: DYKDDDDK) diluted in 100 µl of binding buffer, 1h at 4°C. For the binding reaction, 40 µl of the 50% slurry containing immobilized, HA-GST-tagged v-ATPase subunits were incubated with 10 µl of the eluted FLAG-Ragulator (10% of total purified protein) in 60 µl total supplemented with 1% BSA and 2mM DTT, 1h 30min at 4°C. Samples were then washed 3 times in binding buffer, and subjected to

immunoblotting for FLAG (to reveal bound Ragulator proteins), and for HA (loading control for bait).

In vitro binding of epitope-tagged Raptor to lysosomes

Confluent HEK-293T cells stably expressing FLAG-RagB, plated in 2x15cm dishes, were rinsed once in cold PBS, then scraped, spun down and resuspended in 750ul of fractionation buffer: 50mM KCl, 90mM K-Gluconate, 1mM EGTA, 5mM MgCl2, 50mM Sucrose, 20mM HEPES, pH 7.4, supplemented with 2.5mM ATP, 5mM Glucose and protease inhibitors. Cells were mechanically broken by spraying 4-5 times through a 23G needle attached to a 1ml syringe, then spun down at 2000g for 10min, yielding a post nuclear supernatant (PNS). The PNS was divided into several aliquots and spun at max speed for 15 min in a tabletop refrigerated centrifuge, thus separating the cytosol (the supernatant) from the light organellar fraction (the pellet), which contains the FLAG-RagB lysosomes. This procedure has been shown to yield intact lysosomes, which can be resuspended and used in various functional assays (*3*). Each organellar aliquot was resuspended in 300ul of fractionation buffer, supplemented where required with 1x total amino acids or amino acid esters, 250µM GTP, 100 µM GDP, and small molecules, and incubated for 15 min at 37°C, 400rpm in an Eppendorf Thermomixer (activation step). Meanwhile, a cytosolic preparation was obtained from HEK-293T cells expressing myc-Raptor. Following the 15 min activation step, equal amounts of myc-Raptor cytosol were added to each FLAG-RagB organellar aliquot, and incubated at 37°C, 400rpm in the Eppendorf Thermomixer for further 25 min (binding step). At the end of the binding step, 1.5 volumes of lysis buffer containing 1% Triton X-100 were added to dissolve membranes, along with 35µl of anti-FLAG affinity beads (Sigma). By dissolving the organelles, the addition of Triton X-100 ensured that only the myc-raptor bound to FLAG-RagB was recovered. FLAG-RagB was immunoprecipitated for 2h, followed by immunoblotting for FLAG tag, myc tag, and endogenous proteins.

In an alternative version of this assay, 2x15cm dishes each containing 10^6 HEK-293T cells were transfected with 2 µg each of HAGST-RagC and HA-RagB cloned in the pRK5 expression vector; 48hrs later, cells were starved for amino acids and fractionated as described above to obtain light organellar preparations. Each organellar aliquot was stimulated with amino acid esters for 15 min, then incubated with 0.2-1 µg of highly purified FLAG-raptor for further 25 min. Samples were then supplemented with 1 volume of lysis

buffer containing 1% Triton X-100 and immunoprecipitated with 35µl of Glutathione affinity beads (Pierce), followed by immunoblotting for FLAG tag and HA tag.

In the raptor binding assay to immunoisolated lysosomes, a PNS was generated from HEK-293T cells stably expressing LAMP1 fused to mRFP and to a double FLAG tag (LAMP1-mRFP-FLAG^{X2}). The PNS was divided into aliquots; each sample was diluted to 2ml with fractionation buffer, and subjected to immunoprecipitation with 50µl of anti-FLAG affinity beads for 3h at 4°C. This resulted in selective capture of lysosomes, while all other organelles were discarded through washing in a large excess of fractionation buffer. After 3 washes, the affinity beads-bound lysosomes were resuspended in 300 µl of fractionation buffer supplemented with amino acids or amino acid esters, GTP/GDP and small molecules. Following a 15min activation step, cytosol containing epitope-tagged raptor was added for further 25min at 37°C; finally, samples were washed 3 times in fractionation buffer and subjected to western blotting for FLAG, myc and endogenous proteins.

Measurement of amino acid accumulation inside lysosomes

LAMP1-mRFP-FLAG^{X2} expressing HEK-293T cells were starved for amino acids for 50 minutes in the presence of 2µg/ml cycloheximide, then stimulated for 10 minutes with a mixture containing a 1:100 dilution of native amino acids supplemented with ^{14}C-labeled total amino acids (Perkin Elmer). Typically, 7.5uCi were added to a confluent 15cm dish, yielding a final concentration of 750nM labeled amino acids. Following cell fractionation, lysosomes were immunoisolated as described in the previous section. Where required, anti-FLAG immunoprecipitation was carried out in the presence of 0.5% Triton X-100, or 90 ng/ml Streptolysin O (Sigma) and 1mM DTT. After extensive washing in cold fractionation buffer, FLAG affinity beads with bound lysosomes were transferred to scintillation vials using cut pipet tips, and ^{14}C radioactivity was measured in a scintillation counter. Negative control was provided by an equal amount of FLAG beads incubated with a PNS preparation from wild type HEK-293T cells stimulated with ^{14}C-labeled total amino acids. 4 independent samples were measured for each condition.

RNAi in Drosophila S2 cell

dsRNAs against *Drosophila* lysosomal genes were designed as described in (*4*). To minimize

off-target effects, we used the DRSC tool at http://flyrnai.org/RNAi_find_frag_free.html and excluded regions of 19-mer-or-greater identity to any *Drosophila* transcripts. dsRNAs targeting GFP and dRagC were used as a positive and negative control, respectively.
On day 1, 4 million S2 cells were plated in 6-cm culture dishes in 5 ml of Express Five SFM media. Cells were transfected with 1 μg of dsRNA per million cells using Fugene (Roche). 2 days later, a second round of dsRNA transfection was performed. On day 5, cells were rinsed once with amino acid-free Schneider's medium, and starved for amino acids by replacing the media with amino acid-free Schneider's medium for 1.5 hours. To stimulate with amino acids, the amino acid-free medium was replaced with complete Schneider's medium for 30 minutes. Cells were then washed with ice cold PBS, lysed, and subjected to WB for phospho-T398 dS6K and total dS6K.

S2 cell size measurements

S2 cells were treated with dsRNAs as described above. On day 5, cells were harvested by pipetting in a 4 ml volume, diluted 1:20 with counting solution (Isoton II Diluent, Beckman Coulter), and cell diameters determined using a particle size counter (Coulter Z2, Beckman Coulter) with Coulter Z2 AccuComp software.

Mammalian shRNAs and cDNAs

The sequences of the shRNAs targeting human ATP6V0c are the following (5' to 3')
V0c_1: CCGGGCTCGGCCTCTACGGTCTCATCTCGAGATGAGACCGTAGAGGCCGAGCTTTTT
V0c_2: CCGGTGACGACATCAGCCTCTACAACTCGAGTTGTAGAGGCTGATGTCGTCATTTTT
V0c_3: CCGGGCGCTGCCTATGGCACAGCCACTCGAGTGGCTGTGCCATAGGCAGCGCTTTTT
The sequence of the shRNA targeting human ATP6V1A is the following (5' to 3')
V1A_1:
CCGGCACAGTCTCTATCCAAGTATTCTCGAGAATACTTGGATAGAGACTGTGTTTTTG

Lentiviral shRNAs targeting human RagC and RagD were previously described (*1*).
Human cDNAs for the following genes were purchased from Open Biosystems: ATP6V0D1, ATP6V1D, ATP6V1A, ATP6V1B1, ATP6V1C1, ATP6V1C, ATP6V1E2, ATP6V1H, PAT1, PAT4.

Amino acid starvation/stimulation and ConA/SalA treatments.

HEK-293T cells in 10 cm culture dishes or coated glass cover slips were rinsed with and incubated in amino acid-free RPMI for 50 minutes, and stimulated with a 10X mixture of amino acids or amino acid esters for 10-15 minutes. After stimulation, the final concentration of amino acids in the media was the same as in RPMI. The 10X mixture was prepared from individual powders of amino acids or amino acid esters. $RagA^{+/+}$ and $RagA^{GTP/GTP}$ MEFs were starved for 50 min in amino acid-free RPMI supplemented with 10% fetal calf serum that had been dialyzed against phosphate-buffered saline (PBS) in dialysis cassettes (Thermo Scientific) having a 3500 molecular weight cutoff.

Where drug treatment was performed, cells were incubated with various concentrations of ConA or SalA during the 50 min starvation period and the 10 min stimulation period. In some cases, the drugs were added immediately prior to amino acid stimulation, thus reducing the inhibition period to 12-15 minutes.

Overexpression of amino acid transporters

For co-transfection experiments, 2 million HEK-293T cells were plated in 10 cm culture dishes. 12-16 hours later, cells were transfected with the prk5-based cDNA expression plasmids indicated in the figures in the following amounts: 250 ng myc-PAT1 or myc-PAT4; 100 ng HA-GST-tagged $RagB^{GTP}$; 100 ng HA-GST- tagged $RagC^{GDP}$; 2 ng of FLAG-S6K1. Transfection mixes were taken up to a total of 2 µg of DNA using empty pRK5 vector. 48 hours after transfection, cells were starved and re-stimulated with amino acids, lysed and subjected to immunoprecipitation for FLAG-S6K1 using an anti-FLAG affinity beads for 2h at 4°C. Immunoprecipitates were analyzed by immunoblotting for phospho-T389 S6K1 and total S6K1. Total cell lysates were immunoblotted for HA and myc tags.

Immunofluorescence assays

HEK-293T cells were plated on fibronectin-coated glass coverslips in 35mm tissue culture dishes, at 300,000 cells/dish. 12-16 hours later, the slides were rinsed with PBS once and fixed for 15 min with 4% paraformaldehyde in PBS at RT. The slides were rinsed twice with PBS and cells were permeabilized with 0.05% Triton X-100 in PBS for 5 min. After rinsing twice with PBS, the slides were incubated with primary antibody in 5% normal donkey serum for 1 hr at room temperature, rinsed four times with PBS, and incubated with secondary

antibodies produced in donkey (diluted 1:1000 in 5% normal donkey serum) for 45 min at room temperature in the dark, washed four times with PBS. Slides were mounted on glass coverslips using Vectashield (Vector Laboratories) and imaged on a spinning disk confocal system (Perkin Elmer) or a Zeiss Laser Scanning Microscope (LSM) 710.

Measurement of lysosomal pH

Determination of lysosomal pH was carried out on intact cells by ratiometric imaging of the pH-sensitive dye Oregon Green 514, as described (*3*). One day before the experiment, 1 million HEK-293T cells plated in 35mm culture dishes were fed with 30μg/ml 70KDa Dextran conjugated to Oregon Green 514 (Dx-OG514, Invitrogen). The next day, cells were rinsed 3 times to remove excess dye, and chased for 2h in amino acid-free RPMI; this resulted in the selective accumulation of Dx-OG514 in lysosomes. Where required, cells were stimulated for 15 min with amino acids or amino acid esters at the end of the chase period, or treated with v-ATPase inhibitors. Cells were then detached by pipetting, resuspended in 200μl of a physiological buffer (136mM NaCl, 2.5mM KCl, 2mM $CaCl_2$, 1.3mM $MgCl_2$, 5mM Glucose, 10mM HEPES pH 7.4), and transferred to a black 96-multiwell. Dx-OG514 fluorescence was collected at 530 nm upon excitation at 440nm and 490nm in a Spectramax microplate reader (Molecular Devices).

The 490/440 fluorescence emission ratios were interpolated to a calibration curve. The calibration curve was built by resuspending various cell aliquots in a K^+ isotonic solution (145 mM KCl, 10 mM glucose, 1 mM MgCl2, and 20 mM of either HEPES, MES, or acetate) buffered to pH ranging from 3.5 to 8.0 and containing 10 μg/ml nigericin. The 490/440 ratios were plotted as a function of pH, and fitted to a Boltzmann sigmoid. All measurements were performed in triplicate.

In vitro v-ATPase assays

This assay was modified from (*3*). Two 15cm confluent dishes of HEK-293T cells were incubated overnight with 30μg/ml Dx-OG514. The next day, cells were washed and chased for 2h in serum free DMEM to allow lysosomal accumulation of Dx-OG514. 15 minutes prior to lysis, 1μM FCCP was added to dissipate the lysosomal pH gradient. Cells were then harvested and mechanically broken by spraying 4-5 times through a 23G needle in 750 of fractionation buffer: 50mM KCl, 90mM K-Gluconate, 1mM EGTA, 50mM Sucrose, 20mM

HEPES, pH 7.4, supplemented with 5mM Glucose, protease inhibitor and 1μM FCCP. The resulting PNS was divided into aliquots, and each aliquot was spun down at max speed on a tabletop refrigerated centrifuge, yielding a pellet containing the organellar fraction. Each organellar pellet was resuspended in 180μl of fractionation buffer without FCCP and transferred to a black 96-multiwell. Baseline fluorescence was collected at 530nm upon 490nm excitation in a Spectramax microplate reader at 30 sec intervals for 5 min. To activate the v-ATPase, 5mM ATP and $MgCl_2$ were added to each well, and fluorescence reading was resumed for further 25 min. Lysosomal reacidification by the v-ATPase caused the fluorescence emission of Dx-OG514 to decay exponentially over time. To test for the inhibitory effect of AMP-PNP on v-ATPase activity, 1-10 mM AMP-PNP was added along with 5mM ATP; a concentration-dependent inhibitory effect of AMP-PNP on lysosomal acidification was observed. Once lysosomes had acidified, 1-10 μM FCCP was added to some samples to verify that, under conditions matching the *in vitro* raptor binding assay, rapid dissipation of lysosomal proton gradient occurred.

SUPPLEMENTAL REFERENCES

1. Y. Sancak *et al.*, *Science*, (May 22, 2008).
2. Y. Nemoto, P. De Camilli, *EMBO J* **18**, 2991 (Jun 1, 1999).
3. B. E. Steinberg *et al.*, *J Cell Biol* **189**, 1171 (Jun 28).
4. D. D. Sarbassov *et al.*, *Curr Biol* **14**, 1296 (Jul 27, 2004).

SUPPLEMENTAL TABLE AND FIGURE LEGENDS

Figure S1

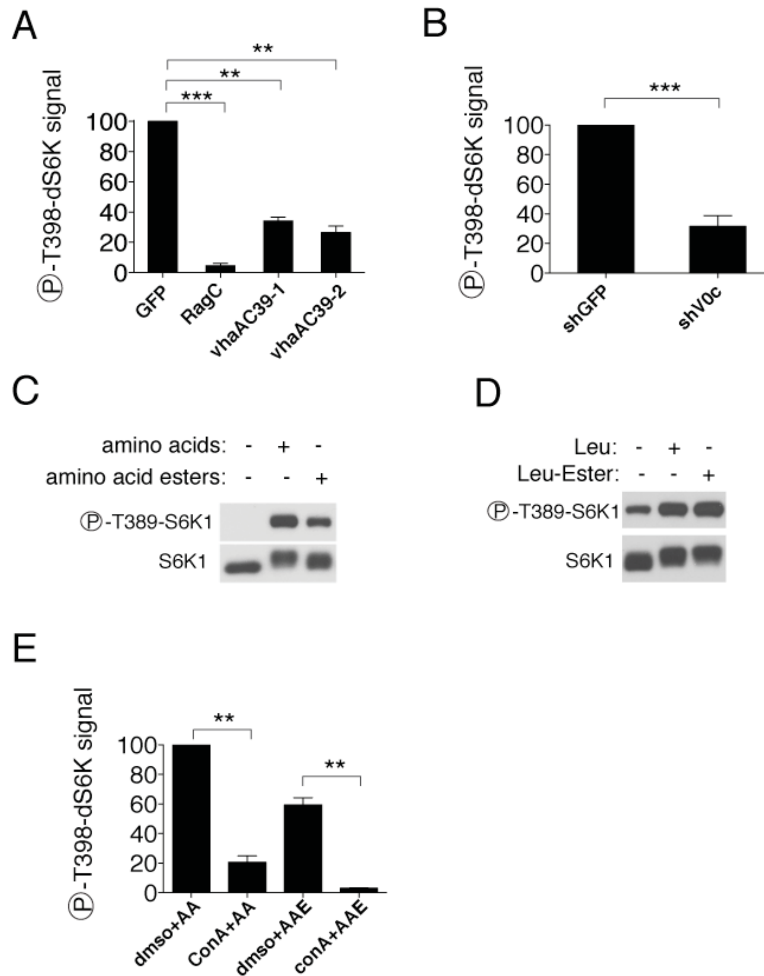
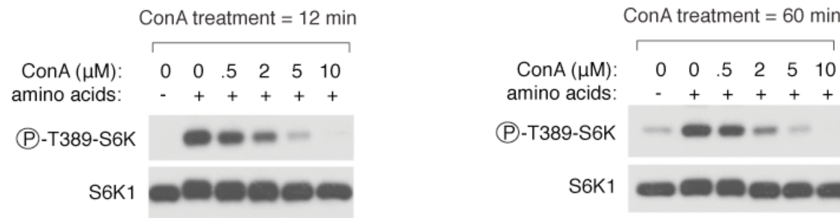


Fig. S1. Requirement for the v-ATPase in mTORC1 activation by amino acids. (**A**) Densitometric analysis of dS6K phosphorylation at T398 following amino acid stimulation in Drosophila S2 cells treated with the indicated dsRNAs. N = 3; $^{**}P < 0.0015$; $^{***}P < 0.0002$. (**B**) Densitometric analysis of S6K phosphorylation at T389 following amino acid stimulation in HEK-293T cells treated with the indicated shRNAs. N = 4; $^{***}P < 0.0003$. (**C**) HEK-293T cells were deprived of amino acids for 50 min and then stimulated with a complete mixture of native amino acids or alcohol esters of amino acids for 10 min. Cell lysates were analyzed for phosphorylation of S6K at threonine 389 (T389). (**D**) HEK-293T cells were deprived of leucine for 50 min and then stimulated with leucine or leucine methyl ester for 10 min. Cell lysates were analyzed for phosphorylation of S6K at T389. (**E**) Densitometric analysis of S6K phosphorylation at T389 following stimulation with amino acids or amino acid esters in HEK-293T cells treated with the indicated chemicals. N = 3; $^{**}P < 0.003$.

Figure S2

A



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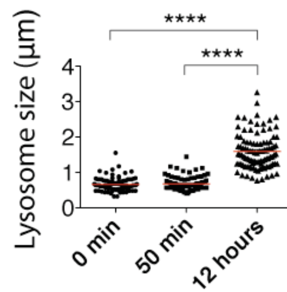

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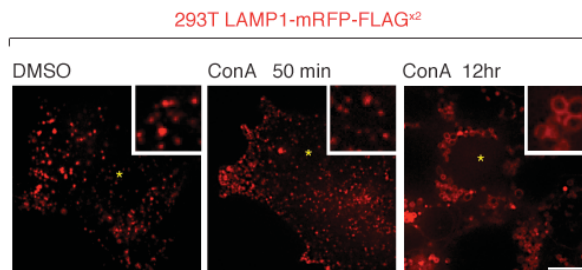

Fig. S2. Time-dependent effect of v-ATPase inhibition on lysosomal morphology. (**A**) S6K1 phosphorylation in HEK-293T cells deprived of amino acids for 50 min and then stimulated for 10 min with amino acids. During the starvation/stimulation period, cells were treated with the indicated concentrations of Concanamycin A (ConA) for 12 minutes (left) or 60 minutes (right). (**B**) Measurement of lysososomal diameter in LAMP1-mRFP-FLAG^{X2} expressing HEK-293T cells that were treated with 2.5 µM ConA for 0 min, 50 min or 12 hrs. N = 100 spots per condition; ****P < 0.0001. Red lines indicate the mean value. (**C**) Representative images from LAMP1-mRFP-FLAG^{X2}-expressing HEK-293T treated with 2.5 µM ConA for the indicated times. Yellow asterisks indicate the position of cell nuclei. Inset shows a higher magnification of a selected field.

Figure S3

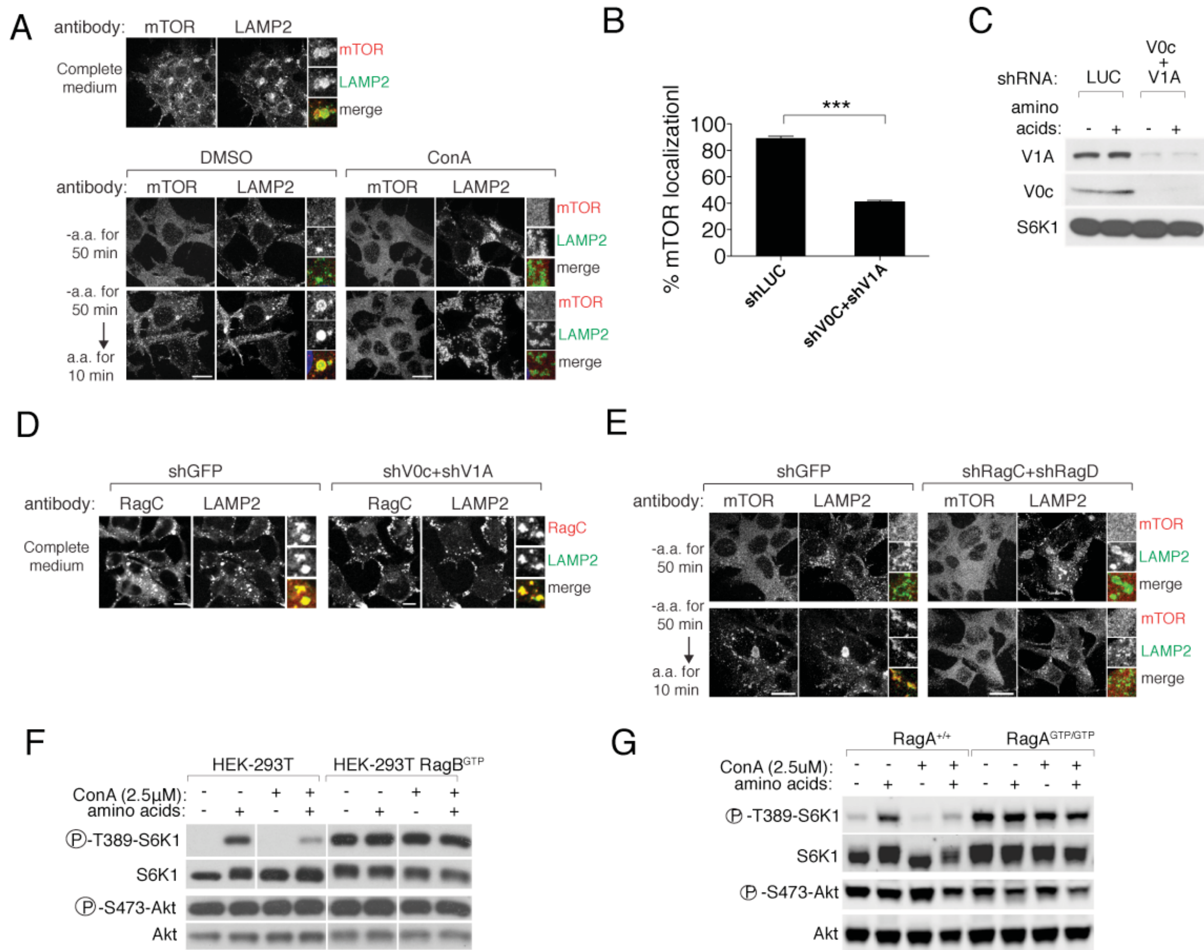

Fig. S3. GTP-bound mutants of RagA and RagB rescue block of mTORC1 lysosomal recruitment and signaling by ConA. (**A**) Immunofluorescence images of HEK-293T cells deprived of amino acids (*top*) or deprived and then stimulated (*bottom*) in the presence of DMSO (*left*) or 2.5 μM ConA (*right*). The mTOR and LAMP2 channels are shown separately. Inset shows a higher magnification of a selected field. In the merge, yellow indicates co-localization. Scale bars represent 10 μm. (**B**) Quantification of cells displaying lysosomal spots of mTOR fluorescence following amino acid stimulation in HEK-293T cells treated with a control shRNA (shLuc), or with shRNAs against V0c and V1A. N = 300 cells per condition from three independent coverslips; ***P < 0.0008. (**C**) HEK-293T treated with control shRNA (shLUC) or with shRNAs against V0c and V1A were lysed and subjected to immunoblotting for the indicated proteins. (**D**) Immunofluorescence images of HEK-293T cells treated with control shRNA (shLUC) or with shRNAs against V0c and V1A and stained for RagC and LAMP2. Scale bars represent 10 μm. (**E**) mTOR/LAMP2 immunofluorescence images of HEK-293T cells treated with control shRNA (shGFP) or with shRNAs against RagC and RagD and deprived of amino acids (*top*) or deprived and then stimulated (*bottom*). Inset shows a higher magnification of a selected field. In the merge, yellow indicates co-localization. Scale bars represent 10 μm. (**F**) S6K1 phosphorylation in control HEK-293T cells and HEK-293T cells stably expressing RagB^{GTP}, which were deprived of amino acids for 50 min in the presence of DMSO or 2.5 μM ConA and then stimulated for 10 min with amino acids. Cell lysates were immunoblotted for the indicated proteins. (**G**) S6K1 phosphorylation in wild-type MEFs (RagA$^{+/+}$) or in MEFs homozygous for the constitutive active RagA Q66L mutant (RagA$^{GTP/GTP}$), deprived of amino acids for 50 min in the presence of DMSO or 2.5 μM ConA and then stimulated for 10 min with amino acids. Cell lysates were immunoblotted for the indicated proteins.

Figure S4

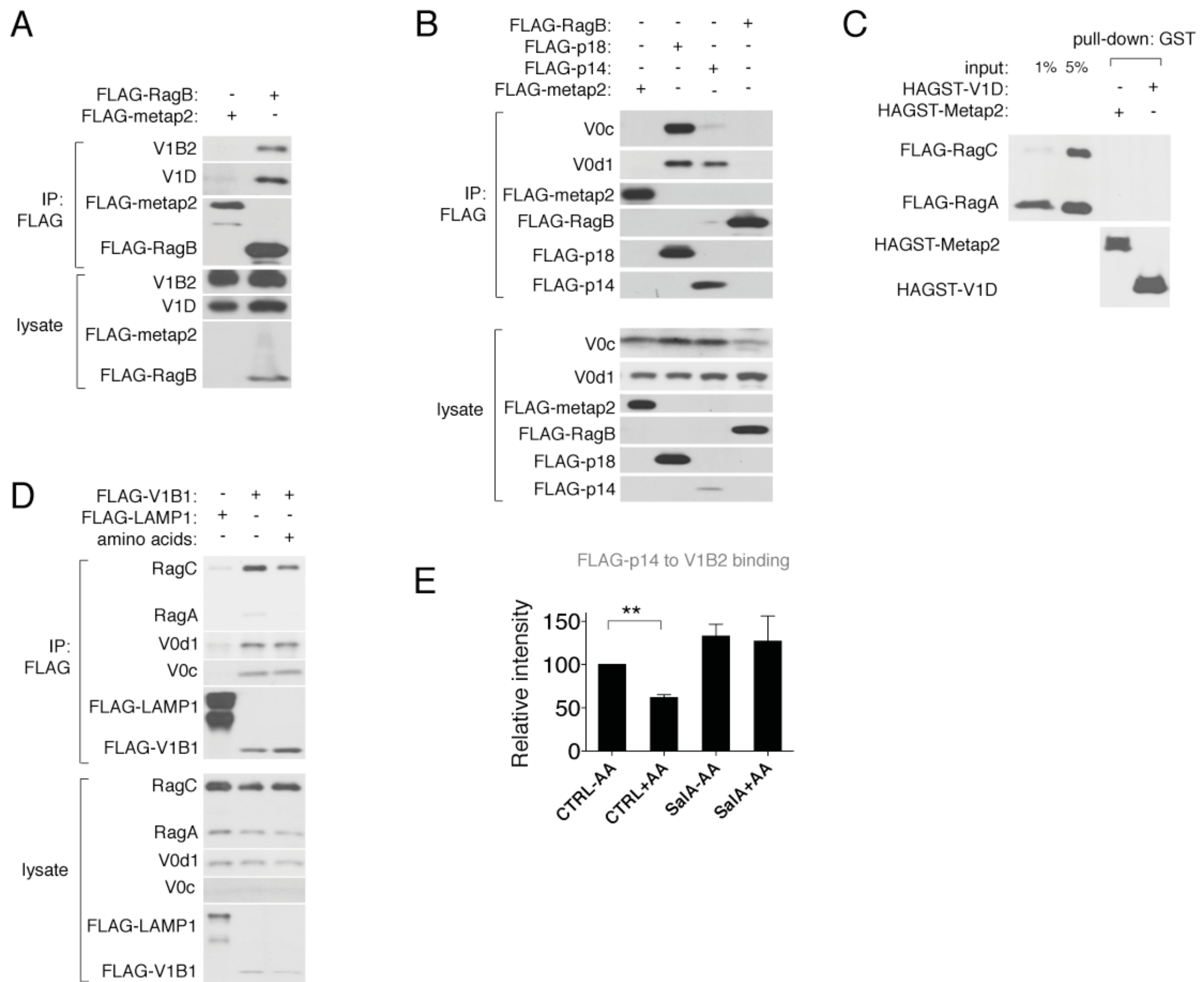

Fig. S4. Interaction between the v-ATPase and the Rag GTPases. (**A**) The Rags binds to the V1 sector. HEK-293T cells stably expressing FLAG-tagged RagB were lysed and subjected to FLAG-immunoprecipitation followed by immunoblotting for V1B2 and V1D. FLAG-Metap2 served as negative control.
(**B**) The Rags do not co-immunoprecipitate with the V0 sector. HEK-293T cells stably expressing FLAG-RagB were lysed and subjected to FLAG-immunoprecipitation followed by immunoblotting for V0c and V0d1. FLAG-Metap2 served as negative control, FLAG-p18 and FLAG-p14 served as positive controls. (**C**) *In vitro* binding assays in which purified FLAG-RagA and FLAG-RagC were incubated with recombinant V1D fused to glutathione S-transferase (HA-GST-V1D). HA-GST-metap2 served as a negative control. (**D**) The V1-Rag GTPase interaction, but not the V1-V0 interaction, is regulated by amino acids. HEK-293T cells stably expressing FLAG-tagged V1B1 were deprived of amino acids for 90 min, or deprived and restimulated with amino acids for 15 min. Following lysis, samples were subjected to FLAG-immunoprecipitation and immunoblotting for RagC and RagA, and for the V0 subunits d1 and c. FLAG-LAMP1 served as negative control. (**E**) Densitometric analysis of FLAG-p14 binding to V1B2 following amino acid starvation, or starvation followed by stimulation, in HEK-293T cells treated with DMSO vs 2.5 μM SalA. N = 3; $^{**}P < 0.003$.

Figure S5

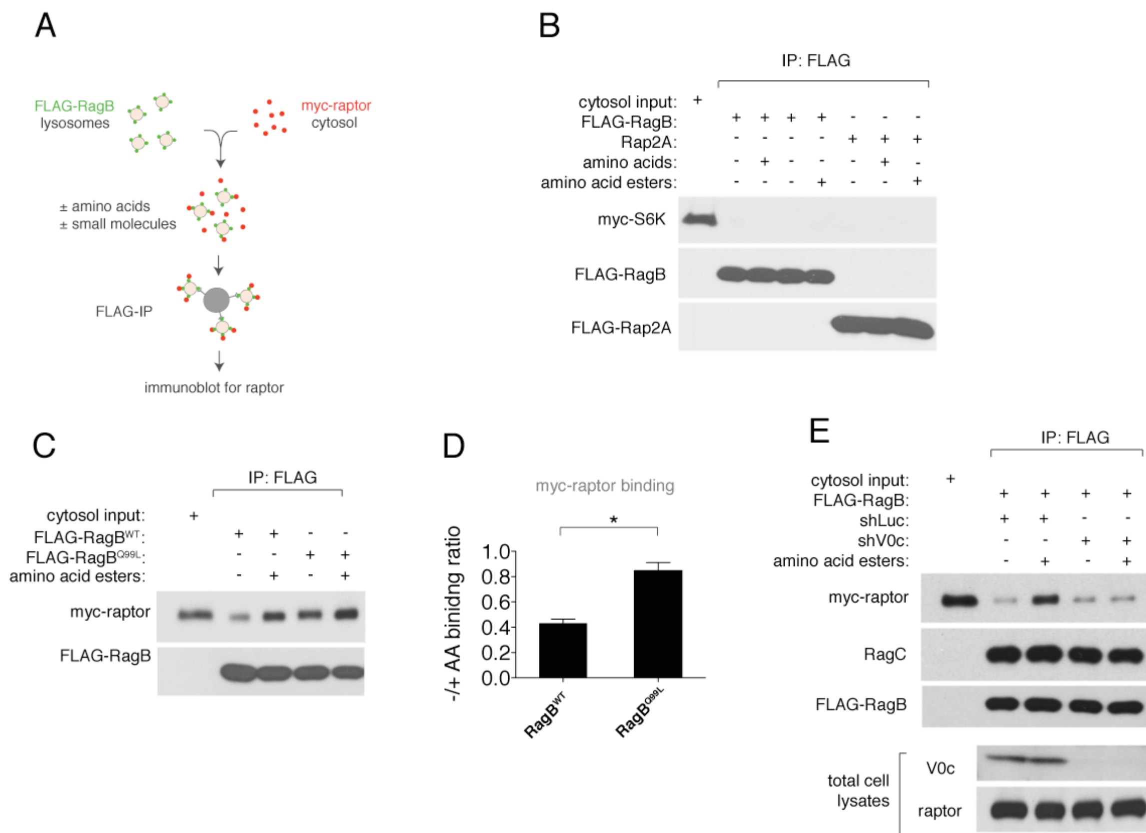

Fig. S5. In vitro assay for lysosomal recruitment of raptor. (**A**) Schematic illustration of the raptor-binding assay to FLAG-RagB containing lysosomes. An organellar fraction from HEK-293T cells stably expressing FLAG-RagB and starved for amino acids is stimulated with amino acids and/or small molecules, followed by incubation with myc-raptor containing cytosol. Following FLAG-immunoprecipitation, myc-raptor bound to FLAG-RagB is detected by immunoblotting. (**B**) Lack of binding of myc-S6K to FLAG-RagB- or FLAG-Rap2A-containing vesicles. Organelle preparations were left unstimulated, or were stimulated with amino acids or amino acid esters and incubated with myc-S6K-containing cytosol. After FLAG immunoprecipitation, immunoblotting for myc tag was performed. (**C**) In vitro binding of myc-raptor to vesicles containing FLAG-$RagB^{WT}$ or constitutively active FLAG-$RagB^{Q99L}$. Organelle preparations were left unstimulated, or were stimulated with amino acid esters, and incubated with myc-raptor-containing cytosol. After FLAG immunoprecipitation, bound myc-raptor was detected by immunoblotting. Notice the higher basal binding of myc-raptor, which was only modestly increased by amino acid esters, in the FLAG-$RagB^{Q99L}$-containing sample. (**D**) Densitometric measurement of myc-raptor binding to FLAG-$RagB^{WT}$ vs FLAG-$RagB^{Q99L}$ containing lysosomes. Bars represent the ratio of myc-raptor bound to starved lysosomes over myc-raptor bound to amino acid ester-stimulated lysosomes. N = 3; *P < 0.02. (**E**) (*Top*) In vitro binding of myc-raptor to lysosomes from FLAG-RagB expressing cells that were treated with a control shRNA (shLuc) or with an shRNA against V0c. (*Bottom*) Total cell lysates from FLAG-RagB expressing cells treated with shLuc or shV0c were immunoblotted for the indicated proteins.

Figure S6

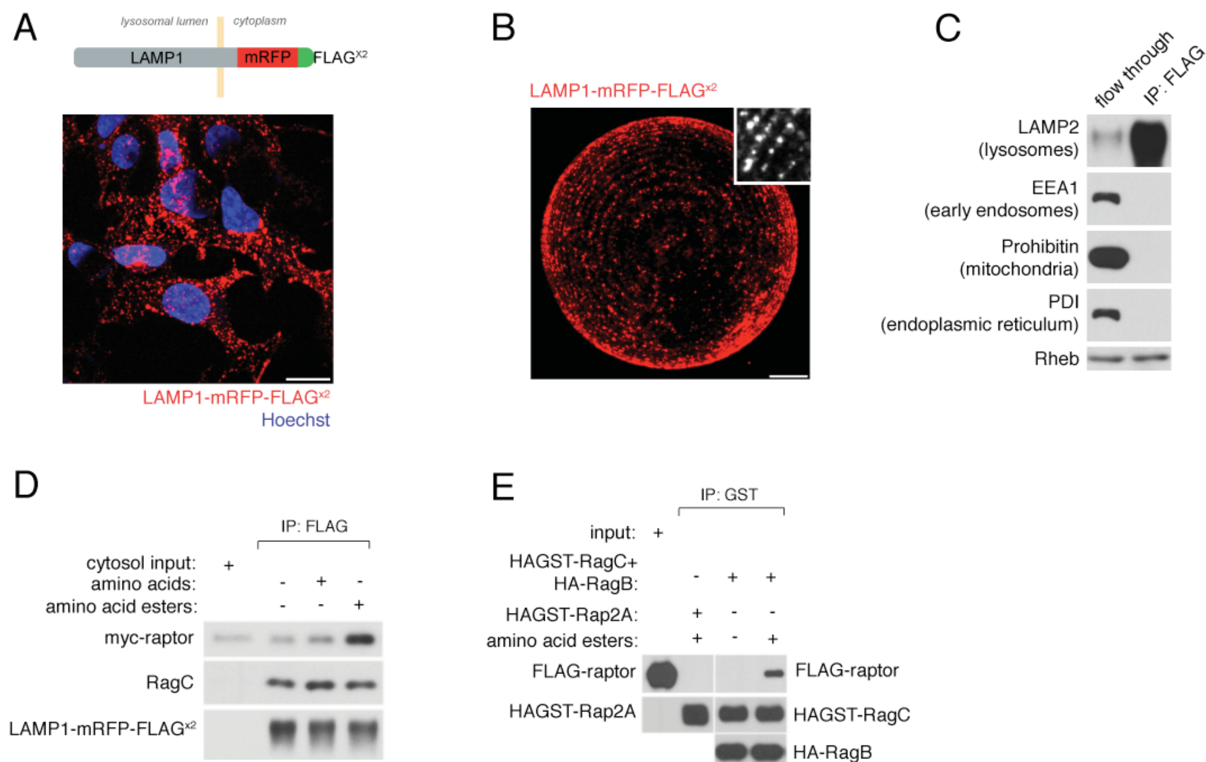

Fig. S6. Recruitment of raptor to immunopurified lysosomes. (**A**) *(Top)* Diagram of the LAMP1-mRFP-FLAG^{X2} construct used for the immunopurification of lysosomes. *(Bottom)* Confocal image of HEK-293T cells stably expressing LAMP1-mRFP-FLAG^{X2}, which is selectively targeted to lysosomes. Scale bar represents 10 μm. (**B**) Confocal projection of an anti-FLAG affinity bead coated with LAMP1-mRFP-FLAG^{X2} positive lysosomes. Inset shows a higher magnification of individual lysosomes, immunoabsorbed to the bead surface. (**C**) Immunoblot analysis of immunopurified lysosomes. Endogenous markers for several organelles are shown. Notice the selective enrichment of the lysosomal marker LAMP2 in the IP lane, while virtually all of Early Endosomal Antigen 1 (EEA1, a marker of early endosomes), Prohibitin (a marker of mitochondria) and Protein Disulfide Isomerase (PDI, a marker of the endoplasmic reticulum) remain in the flow-through. Rheb was detected in both fractions. (**D**) LAMP1-mRFP-FLAG^{X2} positive lysosomes were immunopurified on anti-FLAG affinity beads, activated with amino acids or amino acid esters and incubated with myc-raptor cytosol, followed by immunoblotting for myc tag, FLAG and endogenous RagC. (**E**) Organelles from HEK-293T cells co-expressing HAGST-RagC and HA-RagB were stimulated with amino acid esters and subsequently incubated with highly purified FLAG-raptor, followed by GST immunoprecipitation and immunoblotting for FLAG and HA. Organelles from HEK-293T cells expressing HAGST-Rap2A served as negative control.

Figure S7

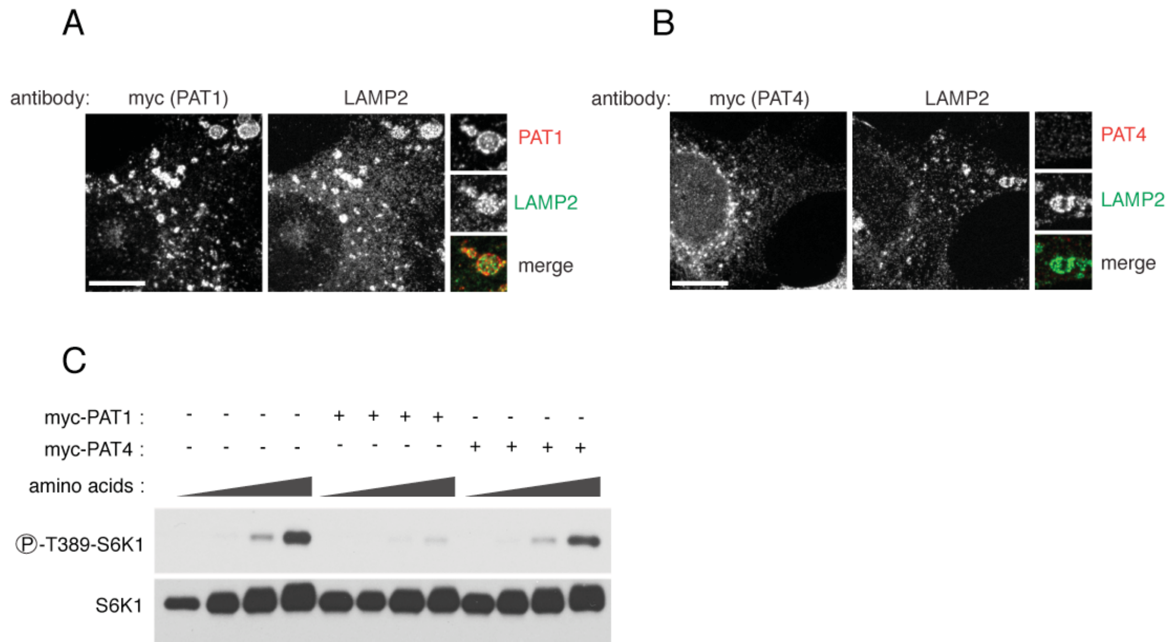

Fig. S7. Overexpression of the PAT1 transporter, but not PAT4, suppresses mTORC1 activation by amino acids. (**A**) Immunofluorescence images of HEK-293T cells transfected with myc-tagged PAT1 and stained for myc tag and LAMP2. Inset shows a higher magnification of a selected field. In the merge, yellow indicates localization of myc-PAT1 to lysosomes. (**B**) Immunofluorescence images of HEK-293T cells transfected with myc-tagged PAT4 and stained for myc tag and LAMP2. Inset shows a higher magnification of a selected field. Notice the absence of myc-PAT4 from LAMP2-positive lysosomes. (**C**) HEK-293T cells transiently expressing FLAG-S6K, FLAG-S6K + myc-PAT1 or FLAG S6K + myc-PAT4 were starved for amino acids for 50 min, or starved and re-stimulated with increasing concentrations of amino acids for 10 min. Following lysis, FLAG-immunoprecipitates were blotted for S6K1 phosphorylation at T389 (see Methods).
In all images, scale bars represent 10 μm.

Figure S8

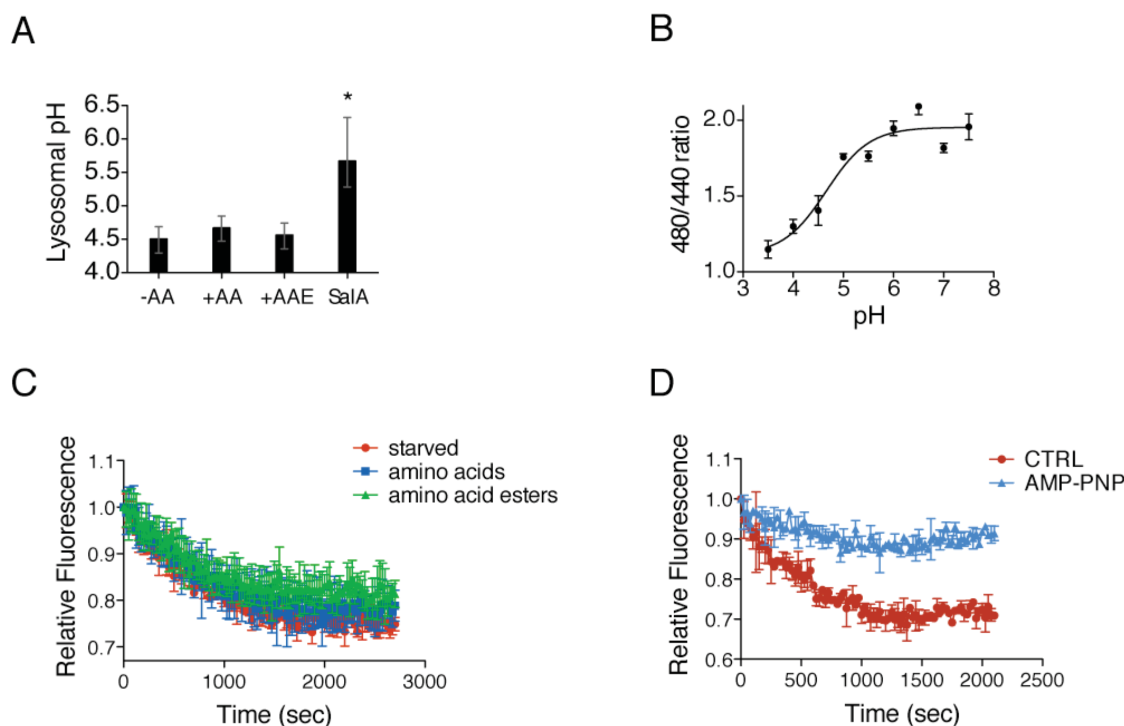

Fig. S8. Spectrofluorimetry-based analysis of lysosomal pH and v-ATPase activity. (**A**) Spectrofluorimetric measurement of lysosomal pH using the 490/440 fluorescence ratio in HEK-293T cells fed overnight with dextran-OG514 (see methods). Prior to pH measurements, cells were starved for amino acids for 50 min, or starved and then stimulated with amino acids or amino acid esters for 10 min, or treated with 2.5μM SalA for 50 min. Asterisk indicates a statistically significant difference (N = 3, $p<0.02$ for all comparisons to SalA treatment). (**B**) Calibration curved obtained by clamping HEK-293T cells at different pH values (see methods), and plotting the 490/440 ratio versus pH. Values were fitted to a Boltzmann sigmoid, and the resulting curve was used to interpolate the 490/440 fluorescence emission values from the experimental points in (**A**). (**C**) In vitro lysosomal acidification assay. Organellar fractions containing lysosomes loaded with dextran-OG514 were isolated from HEK-293T cells. Fractions were resuspended in amino acid-free buffer (red), or in buffer containing native amino acids (blue) or amino acid esters (green); 5mM ATP + $MgCl_2$ were added at the start of the experiment, and quenching of fluorescence emission over time was tracked by spectrofluorimetry. Fluorescence is normalized to the initial value. (**D**) Inhibitory effect of AMP-PNP on v-ATPase activity *in vitro*. V-ATPase-mediated acidification of fractionated lysosomes filled with dextran-OG514 was started by addition of 5mM ATP + $MgCl_2$, in the absence (red) or in the presence (blue) of 10mM AMP-PNP.
In all experiments, each data point represents the mean ± SD of three independent measurements.

Table S1

Requirement for important lysosomal genes in amino acid signaling to mTORC1 as assessed by dsRNA-mediated knock down in Drosophila S2 cells

Drosophila Gene ID	Human Orthologue	Process Involved	dsRNA	Effect on p398-dS6K upon amino acid stimulation
CG8707	RagC	mTORC1 signaling	1	strong suppression
CG5110	MP1	mTORC1 signaling	1	strong suppression
CG14184	p18	mTORC1 signaling	1	strong suppression
CG5189	p14	mTORC1 signaling	1	strong suppression
CG3035	AP3 mu-1	Vesicle traffic	2	no effect
CG11427	AP3 beta-1	Vesicle traffic	2	no effect
CG5625	Vps35	Vesicle traffic	2	no effect
CG4764	Vps39	Vesicle traffic	1	no effect
CG32350	Vps11	Vesicle traffic	2	no effect
CG8454	Vps16A	Vesicle traffic	2	1 dsRNA, minor decrease
CG7146	Vps39	Vesicle traffic	2	no effect
CG18028	Vps41	Vesicle traffic	2	no effect
CG9994	Rab9	Vesicle traffic	2	no effect
CG8385	Arf1	Vesicle traffic	2	no effect
CG8156	Arf6	Vesicle traffic	1	no effect
CG11628	ARNO	Vesicle traffic	1	no effect
CG8200	Flotillin	Lipid rafts	2	no effect
CG5582	CLN3	Lysosomal storage	2	no effect
CG5722	NPC1	Lysosomal storage	2	no effect
CG32672	GABARAP	Autophagy	1	no effect
CG8594	CLCN7	Ion transport	2	no effect
CG3161	ATP6V0C	Ion transport	1	strong suppression
CG1709	ATP6V0A1	Ion transport	2	moderate to strong suppression
CG18617	ATP6V0A2	Ion transport	2	moderate to strong suppression
CG2934	ATP6V0D1	Ion transport	2	strong suppression

dsRNAs against GFP served as positive controls; RagC and Ragulator subunits MP1, p18 and p14 served as negative controls.

Table S1. dsRNA-mediated knock down of selected lysosomal genes in Drosophila S2 cells and their effect on amino acid-induced dTORC1 activation.